



Original Article

Potential Effects of Neonicotinoid Insecticides on Northern Bobwhites

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ABSTRACT Northern bobwhite (*Colinus virginianus*) populations have been declining in Texas, USA, for nearly 3 decades. Reasons for the decline are unclear; however, a variety of factors have been considered, including pesticides and other environmental contaminants. We assessed potential effects of neonicotinoid insecticides on bobwhites by analyzing liver tissues from specimens collected in 3 selected regions of Texas. Fifty-seven bobwhites were collected from the South Texas Plains, Rolling Plains, and Gulf Coast Prairies and Marshes regions during autumn 2014 and spring 2015. Neonicotinoid compounds were detected in trace amounts in the livers of 7 quail, including samples from all 3 field sites and during both collecting periods. Signs of testicular degeneration ($n = 2$) and hepatocellular vacuolation ($n = 10$) were consistent with known results of neonicotinoid intoxication. Overall, we identified evidence of bobwhite exposure to neonicotinoid insecticides, which correlates with a previous study that suggests that neonicotinoid use may be contributing to quail decline in some ecoregions in Texas, particularly the High Plains, Rolling Plains, Gulf Coast Prairies and Marshes, South Texas Plains, and Edwards Plateau. © 2018 The Wildlife Society.

KEY WORDS birds, *Colinus virginianus*, neonicotinoids, northern bobwhite, pesticides, Texas.

Neonicotinoid insecticides have been used in Texas, USA, since the mid-1990s (Elbert et al. 2008). In 2012, 20 Texas counties each applied >2,500 kg of neonicotinoids, and 12 of those applied >5,000 kg (Baker and Stone 2015). Currently, there are 7 different neonicotinoids in the market. Imidacloprid has been the most frequently applied, although clothianidin, thiamethoxam, acetamiprid, dinotefuran, thiacloprid, and nitenpyram are also used (Elbert et al. 2008). Neonicotinoids are effective at controlling many common sucking and chewing insect pests, and used on cereals, fruits, ornamentals, vegetables, cotton, vines, potatoes, and for home, lawn, and veterinary purposes. All compounds act as agonists against nicotinic acetylcholine receptors (nAChRs) in the central nervous system, causing insect paralysis and death (Tomizawa and Casida 2003). They are also systemic, meaning that once applied, they are distributed throughout a plant as it grows, making the plant toxic to feeding insects (Elbert et al. 2008).

Neonicotinoids have systemic properties, and thus are most frequently applied as an insecticidal seed treatment. Since

their registration, the prophylactic use of insecticidal seed treatments has increased dramatically (Douglas and Tooker 2015). Neonicotinoids are highly water soluble and have long half-lives (Fossen 2006, Hladik et al. 2014, Lewis et al. 2015); therefore, application as a seed treatment facilitates their entrance, transport, and persistence in the environment. When applied as a seed dressing, only approximately 5% of the active ingredient reaches the target crop, while the remaining approximately 95% is lost to the environment (Goulson 2014). As a result, neonicotinoids are frequently found in surface waters and detected during and outside of the growing season (Main et al. 2014, Morrissey et al. 2015). In 2012, >160,000 kg of neonicotinoids were applied to Texas crops (Baker and Stone 2015).

Neonicotinoid insecticides are now the most widely used class of insecticide in the world (Jeschke et al. 2011). Neonicotinoids use increased partly because of their high insect specificity and presumably low vertebrate toxicity; however, concerns about their adverse effects on birds and other nontarget organisms have led to scrutiny in recent years (Balani et al. 2011; Blacquière et al. 2012; Lopez-Antia et al. 2013, 2015; Tokumoto et al. 2013; Gibbons et al. 2015).

Use of pesticides in modern agriculture has been linked to declines in bird populations across the globe (Wilson et al. 1999, Benton et al. 2002, Boatman et al. 2004, Mineau and

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Whiteside 2013). In the Netherlands, neonicotinoid levels in surface waters were correlated with declines in farmland birds (Hallmann et al. 2014). Also, poisoning and mortality resulting from the ingestion of neonicotinoid-treated seeds or contaminated insects has been documented (Berny et al. 1999, de Snoo et al. 1999, Bro et al. 2010, Mineau and Palmer 2013). Neonicotinoid residues can persist in field margin vegetation for greater than a year after a field has been planted (Greatti et al. 2006, Krupke et al. 2012). In Texas, neonicotinoid residues were detected in northern bobwhite (*Colinus virginianus*; hereafter, bobwhite) and scaled quail (*Callipepla squamata*) in the Rolling Plains ecoregion during the planting season for winter wheat (Turaga et al. 2015).

Bobwhites are often associated with agriculture (Lusk et al. 2002, Janke and Gates 2013), and can feed on the seeds of agricultural crops (Michael and Beckwith 1955). Adults are primarily granivorous, but also consume green vegetation and invertebrates. Chicks and breeding females are known to increase consumption of invertebrates during breeding and brood-rearing to meet their protein requirements (Larson et al. 2010). Agricultural crops and field margins used by bobwhites for feeding and foraging may be contaminated with neonicotinoid insecticides, which are regularly applied to corn, wheat, sorghum, sunflower, and soybeans, typically in the form of treated seeds (Jeschke et al. 2011). Arthropods selected by bobwhites (i.e., Coleoptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera; Moorman et al. 2013) are targets of neonicotinoids, and bobwhites could

potentially consume these insects after they become contaminated.

Bobwhites have experienced steady long-term declines in most states in the United States (BirdLife International 2016). Causes for bobwhite decline in Texas are not well-established; however, land-use changes resulting from agricultural intensification and urbanization are often proposed as the primary driver of grassland bird decline (Brennan 1991, Brennan and Kuvlesky 2005). Bobwhites are frequently associated with agricultural areas, so they may be affected by neonicotinoid use also because of a reduction in prey abundance during critical periods (e.g., breeding, brood-rearing, and overwintering). Previous work suggested a relationship between neonicotinoid usage and quail population declines (Ertl et al. 2018); therefore, we assessed potential exposure of wild bobwhite quail to neonicotinoids in Texas by measuring residues in tissues and examining potential lesions through histopathological analysis.

STUDY AREA

We selected 3 study sites in areas of high neonicotinoid use in Texas (Fig. 1). The Rolling Plains field site (ROPL) was located near Abilene, Texas, in the central Rolling Plains. This region was dominated by cotton and winter wheat production and cattle ranching (USDA 2010). No ranching activities occurred at the ROPL field site, but the land was managed for white-tailed deer (*Odocoileus virginianus*) production and hunting. Sandy soils dominated this field

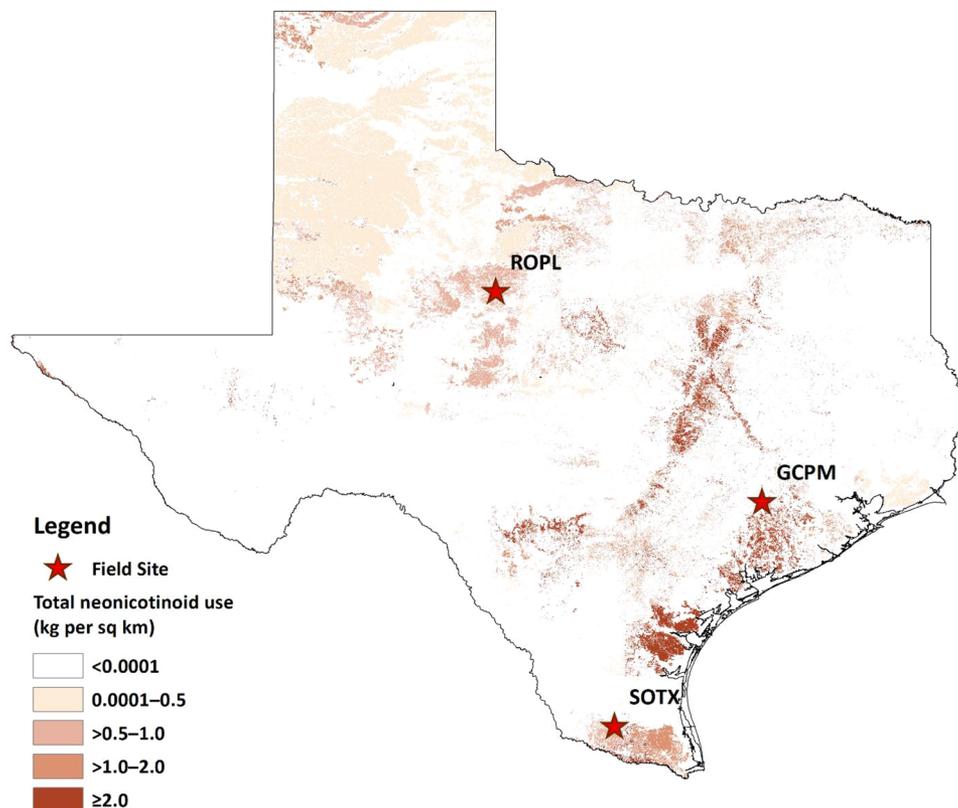


Figure 1. Total estimated neonicotinoid use and study site locations in Texas, USA, for detection of exposure of northern bobwhites during 2014–2015: ROPL (Rolling Plains), GCPM (Gulf Coast Prairies and Marshes), SOTX (South Texas Plains).

site; brush to tree-sized sand shinnery oak (*Quercus havardii*) and various tall grasses grew in abundance, providing habitat for bobwhites. Winter wheat treated with Gaucho[®] (a formulation of imidacloprid; Bayer CropScience LP, Research Triangle Park, NC, USA) was planted in mid-September along sandy roadways and in patches of varying size as supplemental deer feed. Patches often co-occurred with deer feeders containing protein pellets and whole corn, which can be attractive to bobwhites. We used the corn provided as supplemental feed as bait in trapping efforts.

The South Texas Plains field site (SOTX) was located near Edinburg, Texas. This region was dominated by agricultural production, including cotton, citrus, sorghum, corn, sugarcane, sunflower, and vegetables. Huisache (*Acacia farnesiana*) and grasses grow in abundance, providing excellent bobwhite habitat. The SOTX study site was managed for trophy white-tailed deer, exotic antelope, upland game bird hunting, and cattle ranching. This site bordered fields of annual sorghum and sunflower rotation crop. Hunting and trapping efforts were focused solely on patches of woody cover near the agricultural fields. We used locally grown sorghum as bait in our traps, which was provided by the landowner, who used it as supplemental feed for bobwhites.

The Gulf Coast Prairies and Marshes field site (GCPM) was located near Sealy, Texas, in the coastal prairies. The land at the GCPM field site was managed for cattle ranching and upland game bird hunting. Although neonicotinoids were not used by the property owner, cultivated cropland was

located within approximately 1 km of the pastures where we collected specimens. Sampling efforts focused on ungrazed pastures and did not target agricultural areas as with the other 2 field sites. Bobwhites do not receive supplementary feed at the GCPM field site; therefore, hunting was the only method used to collect samples.

METHODS

Sample Collection

Sample collection and analyses were approved by the Texas Parks and Wildlife Department (SPR-0493-605) and the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2014-0183). We collected 27 bobwhite carcasses (7–10/site) during autumn 2014 and 30 (10/site) during spring 2015, for a total of 57 samples (Table 1). We collected bobwhites by hunting on all 3 study sites and by trapping on the ROPL and SOTX study sites. We placed wire funnel traps baited with supplemental feed (corn and sorghum neonicotinoid free) under shrubs or trees bordering agricultural plots or at roost sites.

We euthanized trapped quail via asphyxiation in a CO₂ chamber and conducted a necropsy immediately in the field. We placed quail on a hard, foil-covered surface and determined age (adult or juvenile) by coloration of primary coverts. Bobwhites are sexually dimorphic; thus, we recorded sex based on facial coloration. We recorded body mass with the use of a spring scale and estimated body condition on a

Table 1. Results from chemical and histopathological analyses of tissues of northern bobwhites collected during autumn 2014 and spring 2015 from 3 ecoregions in Texas, USA.

Chemical and histopathological analyses	Ecoregion ^a and year (<i>n</i> samples)					
	SOTX		ROPL		GCPM	
	2014 (10) ^b	2015 (10) ^b	2014 (7) ^c	2015 (10) ^c	2014 (10) ^d	2015 (10) ^d
Neonicotinoids (liver, <i>n</i> = number of detections)						
Imidacloprid	1	1				
Clothianidin	1					1
Acetamiprid	1		2			2
Thiametoxam	1					1
Histopathology						
Few spermatozoa		1T ^c				
Granuloma	1K				1L	2L
Hematopoiesis	1L		2L			1L
Hemosiderin						1L
Hepatitis	1L		1L		1L	
Hepatocellular vacuolation		3L	2L	4L		1L
Hyperplasia		1L		1S		
Intratubular crystals		2K				4K
Lymphoid aggregates				1K, 1L	3L	1K
Mild testicular degeneration		1T				1T
Nematode	1L					
Nephritis		1K				
Tubular degeneration			1K			
Tubular necrosis	1K					
Ureteritis				1K		

^a SOTX = South Texas, ROPL = Rolling Plains, GCPM = Gulf Coast Prairies and Marshes.

^b Sunflower and sorghum.

^c Wheat.

^d Pasture.

^e Letters indicate the following: L = liver, K = kidney, S = spleen, T = testicle.

scale from 1 to 5 using the pectoral muscle (Harper 1999). We recorded all external and internal abnormalities. We extracted liver, kidney, spleen, and gonadal tissue for analysis and stored the remaining carcasses in a cooler with dry ice until we took them to the lab where they were stored in a freezer at -80°C . We incised one mm cross section of liver, whole kidneys, whole testes or ovaries, and whole spleen to allow formalin fixation, and we stored the tissue portions in 90-mL plastic screw-top containers filled with approximately 60 mL of 10% neutral buffered formalin (VWR International, Radnor, PA, USA) and maintained at ambient temperature. We stored the remaining liver tissue and whole crop in Level I EPA quality-assured 60-mL glass screw-top containers (VWR International) on dry ice in the field prior to laboratory storage at -80°C , until chemical analysis. We replaced the foil and sequentially sterilized the utensils with boiling water and acetone between each necropsy.

Chemical Analysis

We analyzed liver and crop content samples for neonicotinoids. We followed methods for sample preparation as described by Xiao et al. (2011) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods as described by Galeano et al. (2013). We extracted liver and crop samples using an accelerated solvent extractor (ASE, Dionex; Thermo Fisher Scientific, Waltham, MA, USA) equipped with 11-mL and 33-mL stainless-steel cells (for liver and crop samples, respectively). We weighed and homogenized the liver tissue with diatomaceous earth (DE; 1:1.25, tissue:DE; Agilent Technologies, Santa Clara, CA, USA) in a 100-mL mortar. We weighed and loosely ground (but not homogenized) crop tissue with diatomaceous earth (1:1.25, tissue:DE) in a 100-mL mortar to prevent samples from clogging the cells. We loaded mixtures into 11-mL or 33-mL cells fitted with a cellulose filter disk (Environmental Express, Atlanta, GA, USA) and $10\ \mu\text{m}$ frit at the bottom. We screwed caps fitted with a $10\text{-}\mu\text{m}$ frit into placement and cells loaded onto the extractor. We collected extracts in 60-mL glass vials. Extraction conditions included pure water as extraction solvent, static extraction time of 5 min, 2 static cycles, extraction temperature at 80°C , and extraction pressure at 10 MPa.

We placed extracts in the refrigerator at 2°C for a minimum of 15 min prior to cleanup. We then loaded the supernatant into a 500-mL Oasis HLB cartridge (Waters Corp., Milford, MA, USA) previously conditioned with 5 mL methanol and 5 mL pure water (Fisher Scientific, Pittsburgh, PA, USA). We passed sample extracts through the columns under a vacuum and rinsed the columns with 5 mL of water and 5 mL of methanol:water (20:80, vol:vol). We eluted analytes with 3 mL of methanol and evaporated the eluate under a gentle stream of nitrogen at 40°C . We topped the remaining residue to 1 mL with methanol:water (30:70 vol:vol) and filtered with a syringe using a $0.2\text{-}\mu\text{m}$ nylon filter (VWR International) into an auto sampler vial.

We used a Waters ACQUITY UPLC/MS system (Waters Corp.). The UPLC was equipped with a binary solvent

manager, sample manager, and column heater, with a tandem quadrupole mass spectrometer equipped with an ESI source. We used an Acquity UPLC BEH Shield RP18 column, $150\text{ mm} \times 2.1\text{ mm}$, $1.7\ \mu\text{m}$ (Waters Corp.). We used nitrogen as a drying gas and nebulizing gas, and argon as the collision gas (Praxair, Bryan, TX, USA). The nitrogen gas flow conditions were 450 and 50 L/hour for desolvation and at the cone, respectively. We set the source block temperature and desolvation temperature at 120°C and 250°C , respectively. Solvents were 0.05% formic acid in H_2O (solvent A) and acetonitrile (solvent B). The gradient was 10% B from 0 to 3 min, 95% B from 2 to 3 min, and 10% B isocratic from 4 to 5 min to allow for column equilibration before the next injection. The flow rate was 0.2 mL/min. We detected neonicotinoids in MS/MS conditions, programming the chromatographic run in the selected reaction monitoring mode (Table S1 in Supporting Information).

We determined limits of detection and quantification by signal-to-noise ratios as described by Galeano et al. (2013). Limits of quantification were as follows: acetamiprid, $0.12\ \mu\text{g}/\text{kg}$; clothianidin, $3.20\ \mu\text{g}/\text{kg}$; imidacloprid, $2.80\ \mu\text{g}/\text{kg}$; and thiamethoxam, $0.50\ \mu\text{g}/\text{kg}$. Limits of detection were approximately 30% below the limits of quantification.

Histopathology Analysis

Liver, kidney, spleen, and gonadal tissues (stored in 10% neutral buffered formalin) were examined for lesions in the Department of Veterinary Pathobiology at Texas A&M University, College Station. Samples were routinely processed for paraffin embedding and slides ($5\text{-}\mu\text{m}$ sections) were stained with hematoxylin and eosin prior to careful examination under a microscope. We recorded all histological and pathological abnormalities observed.

RESULTS

Chemical Analysis

Of the 57 liver samples analyzed, we detected one or more neonicotinoid compounds in 12% ($n=7$) of the samples (Table 1). At the SOTX field site, we detected imidacloprid in the liver of one bobwhite in 2014 and one in 2015, and acetamiprid, clothianidin, and thiamethoxam in another one in 2014 (Table 1). At the ROPL field site, we also detected acetamiprid in 2 bobwhites during 2014; whereas, at the GCPM field site, we detected acetamiprid in one specimen, and acetamiprid, clothianidin, and thiamethoxam in a second specimen, all during spring 2015 (Table 1). Detection levels, however, were below the limit of quantitation in all cases. We did not detect neonicotinoid compounds in any of the crop samples ($n=53$) analyzed.

Histopathology Analysis

Foci of lymphoplasmacytic inflammatory infiltrate and granulomas were present in livers and kidneys of 4 bobwhites (Table 1 and Fig. 2). We detected no infection agents, but observed one intralesional nematode in the liver of one bird. Mild tubulitis with intratubular crystals in the kidney were present in 6 bobwhites. Mild to moderate hepatocellular vacuolation was the most frequent finding (lipid- and glycogen-type; $n=10$; Fig. 2), and mild bile duct hyperplasia

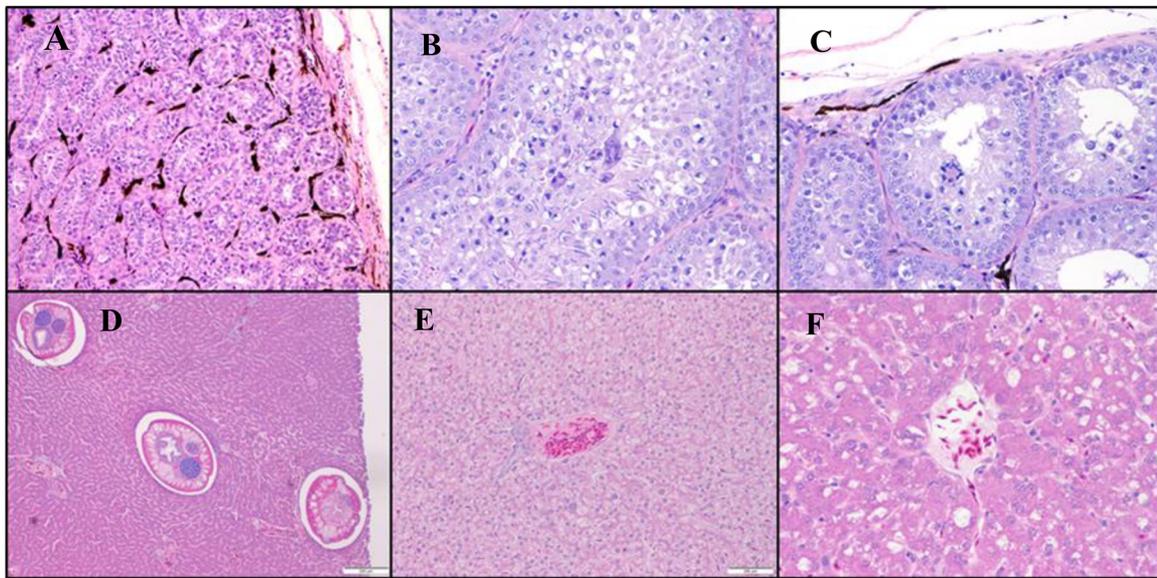


Figure 2. Selected histopathology slides of northern bobwhite tissues collected during 2014–2015 from high-neonicotinoid use areas in Texas, USA. (A) Immature testes; (B, C) multinucleated cells in seminiferous tubules; (D) intralesional nematode; (E) glycogen-type hepatocellular vacuolation; (F) lipid-type hepatocellular vacuolation.

was present in 2 bobwhites. We also identified multinucleated germ cells ($n=2$) in the testes. The spleen of one bird had mild follicular lymphoid hyperplasia. Histological findings in the 7 birds containing neonicotinoid residues included lipid-type hepatocellular vacuolation ($n=1$), autolysis ($n=1$), and hematopoiesis ($n=2$) in the liver, and few intratubular crystals and granulomatous tubulitis in the kidney ($n=1$). Tissues of 2 bobwhites containing neonicotinoids did not show any unusual histopathological lesions.

DISCUSSION

Our analysis indicates that bobwhites were exposed to neonicotinoids in some Texas regions, but observed concentrations were limited and below the limit of quantitation. We detected at least one neonicotinoid compound (imidacloprid, acetamiprid, clothianidin, or thiamethoxam) in 12% of bobwhites collected from the Rolling Plains, Gulf Coast Prairies and Marshes, and South Texas Plains. All neonicotinoid detections were made in liver samples; none of the crop contents contained neonicotinoid residues. Additionally, neonicotinoids were detected in bobwhite tissues during and outside the growing seasons, suggesting that bobwhites may be exposed throughout the year. The low concentrations of neonicotinoids detected in liver could be explained because neonicotinoid compounds are quickly metabolized *in vivo* and do not accumulate in animal tissues (Thyssen and Machemer 1999). Also, we did not detect neonicotinoid residues in the crop contents of all the samples analyzed, indicating that bobwhites were not exposed at the time of collection and bait used in trapping efforts was not contaminated.

Exposure to neonicotinoid mixtures was detected in specimens from the 3 regions, even when there was no

agriculture or neonicotinoid use in the collection area within the GCPM region. The detection of low concentrations of neonicotinoids in 3 bobwhites during spring suggests that exposure to these contaminants can occur during the breeding season, thus potentially affecting reproduction and survival of chicks (Gobeli et al. 2017). Neonicotinoids are known to have synergistic effects on birds when various compounds are applied in mixtures (Morrissey et al. 2015), and elicit sublethal reproductive effects including fewer and fragmented germ cells, reduced fertilization, lower body condition and survival in chicks, impaired embryonic development, reduced clutch size, and delayed egg laying (Balani et al. 2011; Lopez-Antia et al. 2013, 2015; Tokumoto et al. 2013; Pandey and Mohanty 2015). R-selected species, such as bobwhites, invest energy in maximizing their reproductive capacity, but have limited longevity. Annual survival of adult bobwhite is estimated to be as low as 18–30% (Hernandez et al. 2007). Productivity and recruitment are therefore important for maintaining bobwhite populations. However, all birds appeared to be in good body condition, suggesting that food was not limited during the time of collection.

Detection of neonicotinoids in bird tissues coincided with the planting of main agricultural crops in each region; however, at the SOTX field site, neonicotinoid compounds were detected in bobwhite livers in both the autumn and spring. The detection of such compounds in both seasons suggests that neonicotinoids persist in this region throughout the year (Morrissey et al. 2015).

The liver and testes are secondary targets of neonicotinoids (Thyssen and Machemer 1999), which damage tissues through oxidative stress (Tokumoto et al. 2013). Approximately 20% of the bobwhites we collected exhibited evidence of liver or testicular degeneration; however, only one of the

birds, which contained detectable levels of neonicotinoids, displayed tissue degeneration (lipid-type hepatocellular vacuolation) consistent with known results of neonicotinoid toxicity. The absence of detectable compounds in bobwhites exhibiting evidence of neonicotinoid-induced tissue damage could suggest rapid *in vivo* metabolism. Lipid-type hepatocellular vacuolation identified in 10 bobwhites was similar to reported results of Japanese quail (*Coturnix japonica*) that were administered clothianidin and exhibited dose-dependent increases in the number and size of lipid droplets in liver hepatocytes (Tokumoto et al. 2013). Bile duct hyperplasia may be caused by oxidative stress (Bottari et al. 2015) resulting from neonicotinoid toxicity, and was identified in 2 bobwhites in our analysis. It has been shown that the administration of clothianidin to rats (*Rattus* spp.) results in abnormalities in male germ cells (Bal et al. 2012); we found multinucleated germ cells in the seminiferous tubules of 2 bobwhites in our analysis. The lesions we identified that corresponded to known results of neonicotinoid toxicity were likely a secondary toxic result of oxidative stress. Senescence, other pesticides, and various other causes may also induce oxidative stress and could elicit signs of tissue damage similar to what we observed.

Immunosuppression has been linked to a greater susceptibility to parasite infestation and was reported in birds exposed to neonicotinoid compounds (Balani et al. 2011; Köhler and Triebkorn, 2013; Lopez-Antia et al. 2013, 2015). We identified an intralesional nematode in the liver of one bobwhite. Recently, parasitic eyeworms (*Oxyspirura petrowi*) have been found in bobwhites in the Rolling Plains of Texas, and there is still much uncertainty regarding their effects on bobwhite populations (Dunham et al. 2014). It is possible that exposure to neonicotinoids could increase susceptibility of bobwhites to parasite infestation, but further investigations are needed.

Our results indicate that 30% of the bobwhites we collected were either exposed to neonicotinoids in the environment or exhibited evidence of tissue damage corresponding to the known results of neonicotinoid toxicity. These results are in agreement with previous findings, which suggested that neonicotinoids may negatively impact bobwhite populations that frequent cultivated croplands (Ertl et al. 2018). However, there is a need to continue investigations on the use of neonicotinoid insecticides in Texas and their relationship with northern bobwhite population declines. Ultimately, a population-level ecological risk assessment of the impacts of neonicotinoids on bobwhite populations in Texas would be necessary to better establish such cause-effect relationships.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website. Table S1. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) fragmentation of neonicotinoid compounds.